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14. ABSTRACT
We hypothesized that semenogelins, especially semenogelin I (SgI) in the presence of zinc, promote prostate cancer growth via functioning as androgen receptor (AR) coactivators. Using cell lines stably expressing SgI, we investigated biological functions of SgI in prostate cancer. Zinc, without SgI, inhibited cell growth of both AR-positive and AR-negative lines. Co-expression of SgI prevented zinc inhibiting dihydrotestosterone-mediated proliferation of AR-positive cells, whereas SgI and/or dihydrotestosterone showed marginal effects in AR-negative cells. Culture in the conditioned medium containing secreted forms of SgI failed to significantly increase cell viability with or without zinc. Similar effects of SgI overexpression in LNCaP on dihydrotestosterone-induced cell invasion, such as its significant enhancement with zinc, were seen. Overexpression of SgI in LNCaP and CWR22Rv1 cells also augmented dihydrotestosterone-mediated prostate-specific antigen (mRNA, protein) in the presence of zinc. In luciferase assays, SgI showed even slight inhibitory effects at 0 μM zinc and significant stimulatory effects at 100 μM zinc on dihydrotestosterone-enhanced AR transactivation. Using co-immunoprecipitation, we previously demonstrated dihydrotestosterone-induced physical interactions between AR and SgI. These results suggest that intracellular SgI, together with zinc, functions as an AR coactivator and thereby promotes androgen-mediated prostate cancer progression.

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Introduction

Semenogelins, mainly expressed and secreted by the seminal vesicle, are the major structural proteins in human semen containing a high concentration of Zn^{2+}, and their physiological functions have been well characterized. Specifically, semenogelins, upon binding to Zn^{2+}, play an important role in gel-like formation of the semen [1]. After ejaculation, these proteins are degraded into smaller fragments by prostate-specific antigen (PSA), resulting in clotted gel liquefaction and release of the encased spermatozoa [2]. Semenogelins are shown to be expressed in other male genital organs, such as the vas deferens, epididymis, and prostate, as well as in non-genital organs, suggesting their physiological role as modulators of zinc-dependent proteases throughout the body [3,4]. Semenogelin I (SgI) expression has been detected in an androgen-sensitive prostate cancer line LNCaP, which is enhanced by zinc treatment, but not in other prostate cancer lines such as CWR22Rv1, DU145, and PC3 [3,5]. We additionally demonstrated significantly higher levels of nuclear SgI expression in prostatic carcinoma than in non-neoplastic prostatic epithelium or high-grade prostatic intraepithelial neoplasia (PIN), which could also predict biochemical recurrence after radical prostatectomy [5,6]. However, no functional analyses of semenogelins in pathological conditions have been reported and their roles in prostate cancer growth remain uncertain.

We here investigated biological functions of SgI in prostate cancer, mainly using cell line models. The tasks in the approved Statement of Work in this period (months 1-12) would be to characterize semenogelins by testing their effects on the progression of PC *in vitro* (*Task 1*; *1-a – 1-g*).

Body

Prostate Cancer Cell Lines Stably Expressing SgI

Using a lentivirus vector, we generated prostate cancer cell lines stably expressing SgI (*e.g.* LNCaP-SgI, VCaP-SgI, CWR22Rv1-SgI, DU145-SgI, PC3-SgI) and their vector controls. Similarly, silencing of SgI was achieved via short hairpin RNA (shRNA) (*e.g.* LNCaP-control-shRNA, LNCaP-SgI-shRNA). Overexpression or down-regulation of SgI protein in these stable cell lines was then confirmed (figure not shown).

Effects of SgI on Prostate Cancer Cell Proliferation

To see if SgI affects prostate cancer cell proliferation, we first performed MTT (methylthiazolyldiphenyl-tetrazolium bromide) assay in the stable cells. Each subline was cultured for 4 days in the presence or absence of dihydrotestosterone (DHT) (1 nM) and zinc (100 μM). As expected, zinc treatment significantly inhibited the growth of all control lines (Figure 1; 21-45% decrease; lanes 1 *vs.* 2) except LNCaP-V. In AR-positive CWR22Rv1-derived cells (Figure 1A), DHT increased the growth by 12-13% without zinc treatment (lanes 1 *vs.* 3 and 5 *vs.* 7). In the presence of zinc, DHT showed a similar induction rate in CWR22Rv1-V (14% increase; lanes 2 *vs.* 4), whereas overexpression of SgI resulted in a statistically significant increase in the growth rate

(27%; lanes 6 *vs.* 8; p=0.034). Thus, zinc only marginally decreased cell growth of CWR22Rv1-SgI (lanes 5 *vs.* 6 and 7 *vs.* 8). In LNCaP cells with endogenous SgI (LNCaP-V; Figure 1B), zinc treatment did not decrease, rather marginally increased, the growth in the absence (lanes 1 *vs.* 2) or presence (lanes 3 *vs.* 4) of DHT. DHT increased the growth of LNCaP-V without (62%; lanes 1 *vs.* 3; p=0.009) or with (52%; lanes 2 *vs.* 4; p=0.014) zinc as well as that of LNCaP-SgI without (66%; lanes 5 *vs.* 7; p=0.036) or with (82%; lanes 6 *vs.* 8; p=0.018) zinc. Thus, co-expression of SgI in the presence of zinc appeared to induce androgen-mediated proliferation of AR-positive prostate cancer cells and, more importantly, protected the cells from cytotoxic effects of zinc. In AR-negative PC3-derived (Figure 1C) and DU145-derived (Figure 1D) cells, DHT treatment and SgI overexpression showed only marginal effects on their growth (<10% changes).

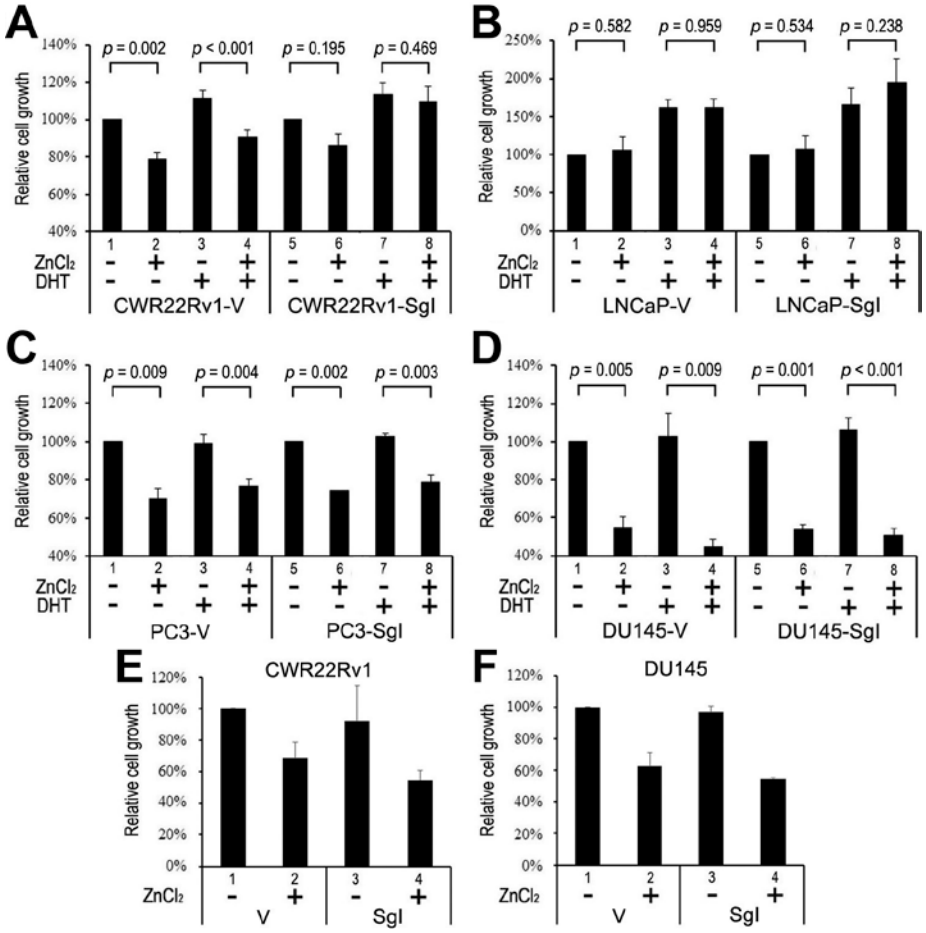

Figure 1. Cell viability of prostate cancer lines stably expressing SgI. CWR22Rv1-V/SgI (**A**), LNCaP-V/SgI (**B**), PC3-V/SgI (**C**), and DU145-V/SgI (**D**) were cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS in the presence or absence of 100 μM zinc and 1 nM DHT for 96 hours. CWR22Rv1 (**E**) and DU145 (**F**) were cultured in conditioned medium (containing 10% normal FBS) derived from CWR22Rv1-V/SgI culture in the presence or absence of 100 μM zinc for 96 hours. Proliferation was assayed with MTT, and growth rates are presented relative to cell number in respective lines with mock treatment [lanes 1 (**A-F**) and 5 (**A-D**); set as 100%]. Each value represents the mean + SD of at least three determinations.

Because semenogelins are secreted proteins [1], we further tested whether secreted forms of SgI induced prostate cancer cell proliferation. MTT assay was again performed in CWR22Rv1 (Figure 1E) and DU145 (Figure 1F) cells incubated in the conditioned medium derived from CWR22Rv1-V/SgI culture. In these parental lines, the secreted form of SgI did not significantly affect cell viability in the absence (lanes 1 *vs.* 3) or presence (lanes 2 *vs.* 4) of zinc.

To investigate how SgI affects cell proliferation, we performed TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay and flow cytometry. However, SgI in the presence or absence of zinc did not significantly change apoptotic indices or G0/G1 population in LNCaP sublines.

Effects of SgI on Prostate Cancer Cell Invasion

To investigate whether SgI promotes tumor invasion, a transwell invasion assay was performed in the stable LNCaP lines (Figure 2). DHT similarly induced cell invasion of LNCaP-V without (35% increase; lanes 1 *vs.* 2; p=0.042) or with (48% increase; lanes 3 *vs.* 4; p=0.009) zinc or LNCaP-SgI without zinc (48% increase; lanes 5 *vs.* 6; p=0.026). In contrast, in LNCaP-SgI with zinc, the invasiveness was more significantly increased by DHT (2.8-fold over mock treatment; lanes 7 *vs.* 8; p=0.006). Thus, significant induction of the DHT-mediated invasive properties by endogenous SgI (lanes 2 *vs.* 4; 19% increase) or exogenous SgI overexpression (lanes 6 *vs.* 8; 88% increase) with versus without addition of zinc was seen.

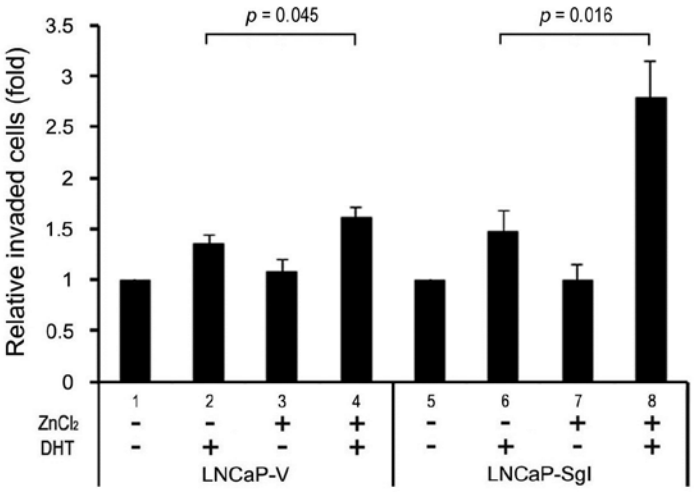

Figure 2. Cell invasion of prostate cancer lines stably expressing SgI. LNCaP-V/SgI cells cultured in the Matrigel-coated transwell chamber for 36 hours in the presence or absence of 300 μM zinc and 1 nM DHT were used for transwell assay. The number of invaded cells in five random fields was counted under a light microscope, using a 40x objective. Invasion ability is presented relative to that in each cell line with mock treatment (lane 1 or 5; set as 1-fold). Each value represents the mean + SD of at least three independent experiments.

Effects of SgI on PSA Expression in Prostate Cancer Cells

We next determined whether SgI regulated the expression of PSA, an androgen-inducible AR target and also known to proteolyze SgI in semen [1,2], in prostate cancer cells. A quantitative RT-PCR showed that DHT treatment, in the absence of additional zinc, increased endogenous *PSA* expression over mock treatment by 3.4-fold (lanes 1 *vs.* 2; p<0.001)/3.8-fold (lanes 5 *vs.* 6; p=0.009) in LNCaP-V/SgI (Figure 3A),

respectively. In the presence of 300 μM zinc, DHT increased *PSA* expression by 4.7-fold (lanes 3 *vs.* 4; *p*=0.004)/7.1-fold (lanes 7 *vs.* 8; *p*=0.003) in LNCaP-V/SgI, respectively. The difference in DHT-mediated *PSA* expression in LNCaP-SgI with versus without zinc was also statistically significant (lanes 6 *vs.* 8; 1.8-fold). Similarly, western blots in CWR22Rv1 cells cultured with 100 μM zinc (Figure 3B) and LNCaP stable cells cultured with 300 μM zinc (Figure 3C) showed that overexpression of SgI resulted in considerable increases in DHT-mediated PSA expression. However, no significant additive effects of SgI on PSA protein expression were seen in these cell lines when cultured without additional zinc (figure not shown).

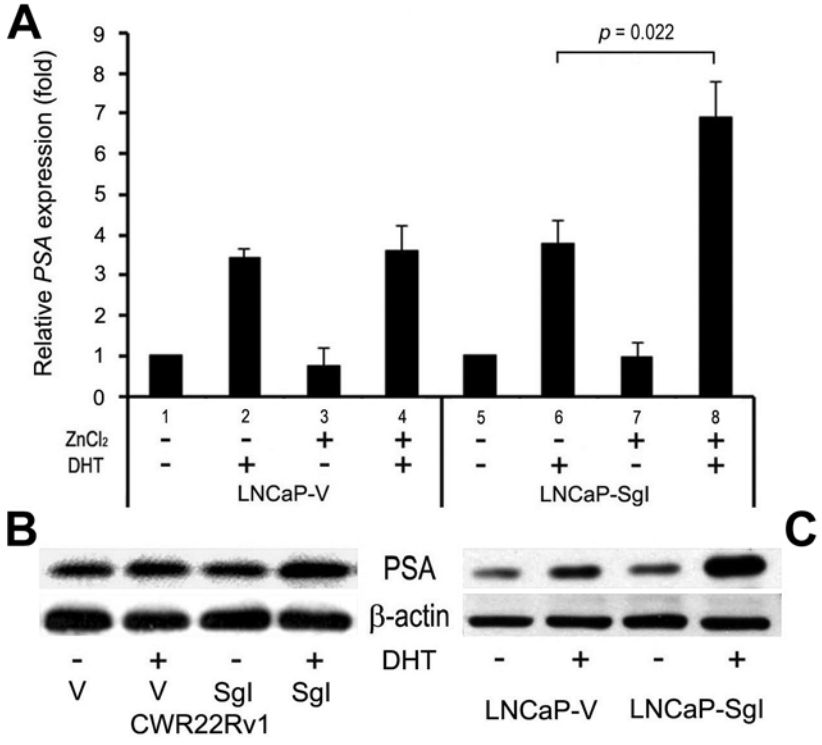

Figure 3. PSA expression in prostate cancer lines stably expressing SgI. (**A**) LNCaP-V/SgI cells cultured in phenol red-free medium supplemented with 5% charcoal-stripped fetal bovine serum (FBS) in the presence or absence of 300 μM zinc and 1 nM DHT for 48 hours were subjected to a quantitative RT-PCR. Expression of *PSA* gene was normalized to that of *GAPDH*. Transcription amount is presented relative to that of mock treatment in each cell line (lane 1 or 5; set as 1-fold). Each value represents the mean + SD from at least three independent experiments. CWR22Rv1 cells (**B**) transiently transfected with pSG5 or pSG5-SgI were cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS in the presence or absence of 100 μM zinc and 1 nM DHT for 48 hours, and LNCaP-V/SgI cells (**C**) were similarly cultured with 300 μM zinc ± 1 nM DHT for 48 hours, as indicated. Cell extracts were then analyzed on western blots, using an antibody to PSA (33 kDa) or β-actin (92 kDa).

Enhancement of AR Transcriptional Activity by SgI in Prostate Cancer Cells

To assess the effect of SgI on androgen-mediated AR transactivation, luciferase activity was determined in PC3 cells transfected with AR, SgI, and an androgen response element-reporter plasmid, and treated with different concentrations of zinc and 1 nM DHT. DHT increased AR transcription by 17-fold (0 μM zinc; Figure 4A), 12-fold (15 μM zinc; Figure 4B), and 10-fold (100 μM zinc; Figure 4C), as compared with respective mock treatments. Thus, zinc reduced androgen-enhanced AR transactivation in a dose-dependent manner. SgI showed a slight inhibitory effect (15% decrease at 0 μM zinc; Figure 4A) or a slight stimulatory effect (31% increase at 15 μM zinc; Figure 4B) on DHT-induced AR transcription. In contrast, in the presence of 100 μM zinc, SgI further

induced DHT-mediated AR transcription by 3.2-fold (Figure 4C). Induction of zinc/DHT-mediated AR transcription by SgI (2.1-fold) was confirmed in CWR22Rv1, while SgI did not significantly affect AR transactivation without additional zinc (8% decrease) (Figures 4D & 4E). These results suggest that SgI functions as an AR coactivator in the presence of zinc in prostate cancer cells.

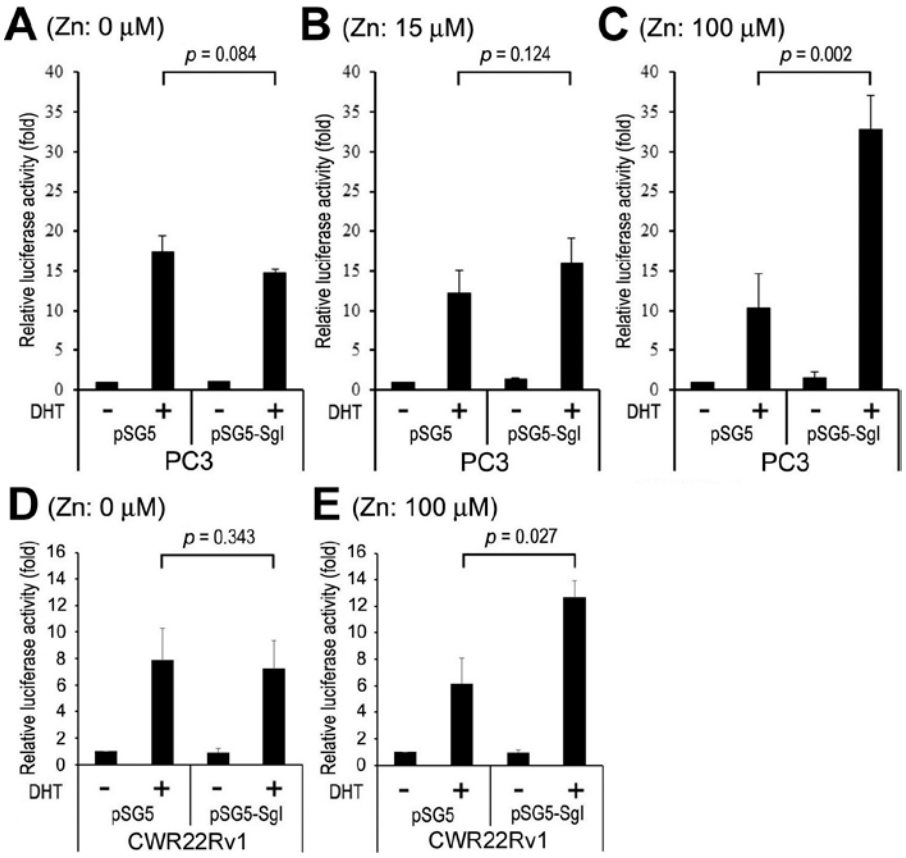

Figure 4. The effects of SgI on AR in prostate cancer cells. PC3 cells were co-transfected with pSG5-AR, MMTV-Luc, pRL-TK, and either pSG5 or pSG5-SgI (AR : SgI = 1 : 5), and cultured in phenol red-free medium supplemented with 5% charcoal-stripped FBS along with mock (ethanol), zinc [(**A**) 0 μM; (**B**) 15 μM; (**C**) 100 μM], and/or 1 nM DHT for 24 hours. Similarly, CWR22Rv1 cells were co-transfected with MMTV-Luc, pRL-TK, and either pSG5 or pSG5-SgI, and treated with mock (ethanol) or 1 nM DHT in the absence (**D**) or presence (**E**) of 100 μM zinc for 24 hours. The luciferase activity is presented relative to that of mock treatment (first lanes; set as 1-fold). Each value represents the mean + SD of at least three determinations.

Key Research Accomplishments

1. (for *Tasks 1-a* & *1-b*) Prostate cancer cell lines stably expressing SgI or SgI-shRNA were established.

2. (for *Tasks 1c* & *1-e*) SgI in the presence of zinc was found to induce androgen-mediated proliferation of AR-positive prostate cancer cells and protect the cells from cytotoxic effects of zinc.

3. (for *Tasks 1d*) SgI in the presence of zinc was found to induce androgen-mediated invasion of AR-positive prostate cancer cells.

4. (for *Tasks 1f*) SgI in the presence of zinc was found to enhance androgen-mediated

AR transactivation in prostate cancer cells.

5. (for *Tasks 1g*) SgI in the presence of zinc was found to induce androgen-mediated PSA expression in AR-positive prostate cancer cells.

Reportable Outcomes

Ishiguro H, Izumi K, Zheng Y, Kashiwagi E, Kawahara T, Miyamoto H: Semenogelin I promotes prostate cancer cell growth via functioning as an androgen receptor coactivator and protecting against zinc cytotoxicity. American Urological Association 109th Annual Meeting, May 2014, Orlando, Florida [J Urol 191 (Suppl 4S): e325, 2014; please see Appendix].

A manuscript presenting the data included in this report has also been submitted for publication.

Conclusion

Our current data indicating that intracellular SgI functions as an AR coactivator and promotes the growth of prostate cancer cells provide its novel role in tumor progression. Particularly, SgI protects the cells against zinc cytotoxicity, which may explain the enigma of high-level zinc accumulation in prostate cancer tissue. Further functional analyses of SgI *in vivo* as well as mechanistic studies are necessary to determine their biological significance in prostate cancer.

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Appendix

Ishiguro H, Izumi K, Zheng Y, Kashiwagi E, Kawahara T, Miyamoto H: Semenogelin I promotes prostate cancer cell growth via functioning as an androgen receptor coactivator and protecting against zinc cytotoxicity. *J Urol* 191 (Suppl 4S): e325, 2014.

and severity of tumours in PTEN conditional knockout mice with prostate-specific deletion of PTEN, correlates with the estrogen sensitivity of each lobe of the prostate. Therefore, we hypothesized that this model could be used to study the role of ERα in prostate cancer progression.

METHODS: Immunohistochemistry and stereology were used to quantify ERα and Ki67 expression in PTEN null mice. To assess the functional role of ERα, a cell line derived from a PTEN null tumour was treated with shRNA or TPSF, a non-competitive ERα antagonist. Rescue experiments with expression constructs for either full length ERα, capable of genomic and non-genomic actions, or membrane-only ERα, only able to trigger rapid non-genomic signalling, were used to determine the mechanism underlying ERα-regulated proliferation.

RESULTS: There was a dramatic increase in ERα expression in prostate tumours of PTEN null mice compared with normal prostates of control animals. Within the PTEN null prostate, there was a consistent pattern of ERα expression: low in benign glands, moderate in tumours within the dorsal, lateral and ventral lobes, and high in tumours within the anterior prostate. This pattern significantly correlated with the levels of the proliferative marker Ki67. There was also a significant correlation between ERα and Ki67 within individual malignant glands in the anterior prostate. In vitro knockdown of ERα attenuated the proliferation of PTEN null cells as did treatment with TPSF. Loss of ERα reduced the activity of both the PI3K and MAPK pathways and decreased MYC levels. This effect was reversed by re-expressing full-length or membrane-only ERα.

CONCLUSIONS: Collectively, these results demonstrate that ERα drives the proliferation of prostate cancer cells through classical genomic and rapid non-genomic signalling.

Source of Funding: ML and LF are Movember Young Investigators funded by the Prostate Cancer Foundation of Australia's Research Program.

MP31-08

SEMENOGELIN I PROMOTES PROSTATE CANCER CELL GROWTH VIA FUNCTIONING AS AN ANDROGEN RECEPTOR COACTIVATOR AND PROTECTING AGAINST ZINC CYTOTOXICITY

Hitoshi Ishiguro, Baltimore, MD; Koji Izumi, Yi Li, Rochester, NY; Yichun Zheng, Eiji Kashiwagi, Takashi Kawahara, Hiroshi Miyamoto, Baltimore, MD*

INTRODUCTION AND OBJECTIVES: A seminal plasma protein, semenogelin I (SgI), contributes to semen clotting, upon binding to Zn^{2+}, and can be proteolyzed by prostate-specific antigen (PSA) to release the encased spermatozoa after ejaculation. In contrast to the well-recognized physiological actions of semenogelins, their role in human malignancies remains poorly understood. We have demonstrated that SgI is overexpressed in prostate cancer tissues and its expression is enhanced by zinc treatment in LNCaP cells. In the current study, using cell lines stably expressing SgI, we investigated its biological functions in prostate cancer.

METHODS: We assessed the effects of SgI, in conjunction with zinc and androgen, on cell growth and androgen receptor (AR) in prostate cancer lines, using western blotting, MTT assay, transwell invasion assay, luciferase assay, and co-immunoprecipitaion assay.

RESULTS: Even though SgI is a secreted protein, immunoblots detected signals in conditioned medium only after culturing SgI-overexpressing cells, but not control LNCaP with endogenous SgI, suggesting that prostate cancer cells do not generally secrete a large amount of SgI. Zinc, without SgI, inhibited cell growth of both AR-positive and AR-negative lines. Co-expression of SgI induced dihydrotestosterone (DHT)-mediated proliferation of AR-positive cells when cultured with zinc, whereas SgI and/or DHT showed marginal effects in AR-negative cells. Similarly, SgI enhanced DHT-induced cell invasion only in the presence of high-level zinc. Moreover, overexpression of SgI induced DHT-mediated PSA expression in cancer cells, whereas SgI showed marginal induction without DHT. In a reporter gene assay, SgI showed a slight inhibitory effect (15% decrease) at 0 μM zinc, a slight stimulatory effect (31% increase) at 15 μM zinc, or a significant stimulatory effect (3.2-fold) at 100 μM zinc on DHT-enhanced AR transactivation. Co-immunoprecipitation then demonstrated DHT-induced physical interactions between AR and SgI.

CONCLUSIONS: We show molecular evidence indicating that cellular SgI, as a new AR coactivator, enhances the transcriptional activity of the receptor in the presence of high levels of zinc and promotes androgen-mediated prostate cancer progression. Our results may also provide an underlying reason why prostate cancer tissue contains relatively high levels of zinc which by itself shows an inhibitory effect on tumor growth.

Source of Funding: Department of Defense

MP31-09

IDENTIFICATION OF A RETRO-TRANSPOSON DERIVED GENE ASSOCIATED WITH PROGRESSION TO NEUROENDOCRINE PROSTATE CANCER.

Shusuke Akamatsu, Alexander Wyatt, Dong Lin, Summer Lysakowski, Fan Zhang, Soojin Kim, Ladan Fazli, Vancouver, Canada; Himisha Beltran, Mark Rubin, New York, NY; Amina Zoubeidi, Yuzhuo Wang, Colin Collins, Martin Gleave, Vancouver, Canada*

INTRODUCTION AND OBJECTIVES: The treatment of castration resistant prostate cancer has dramatically improved with the recent development of potent androgen receptor (AR) pathway inhibitors. However, stronger AR pathway inhibition appears to be driving resistance mechanisms that are independent of the AR axis, the most recognized of which is neuroendocrine prostate cancer (NEPC). To date, few genes have been associated with progression to NEPC. We developed a patient-derived xenograft model of NEPC trans-differentiation: a hormone-naïve adenocarcinoma that upon AR-blockade initially regresses, but rapidly relapses as NEPC. In this study, we carried out longitudinal expression profiling of xenograft tumors during the trans-differentiation process to identify genes associated with tumor cell survival post-castration and the development NEPC.

METHODS: Gene profiling of xenografts collected at different time points during the trans-differentiation were compared to data sets of human NEPC. Immunohistochemistry was performed using clinical NEPC samples. Loss of function studies were carried out using siRNA and shRNA in cell growth (WST-8), invasion (Boyden chamber) and migration (scratch) assays.

RESULTS: We identified a retro-transposon derived gene, Paternally Expressed 10 (PEG10), to be highly expressed during the early trans-differentiation stage and also in clinical NEPC. We confirmed at the protein level that PEG10 is up-regulated post-castration and further significantly elevated in terminal NEPC. PEG10 was highly expressed within NEPC foci of clinical samples. Knockdown of PEG10 in prostate cancer (PC) cells induced apoptosis and G0/G1 arrest, and also attenuated invasion and migration. We found PEG10 knockdown inhibited invasion and migration induced by TGF-β, and modulated response of the cells to TGF-β, resulting in decreased phosphorylation of Smad2 and Smad3, decrease in SBE4 luciferase reporter activity, and inhibition of Snail and Zeb1 induction. Collectively, these data show that PEG10 promotes PC cell growth, and also cooperates with TGF-β to promote invasion and migration of PC cells, conferring aggressive phenotype to these cells.

CONCLUSIONS: PEG10 is a gene associated both with growth and invasion of NEPC, and is a potential novel therapeutic target for the treatment of NEPC.

Source of Funding: None